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Hydrogen Peroxide– and Peroxynitrite-Induced Mitochondrial DNA Damage and Dysfunction in Vascular Endothelial and Smooth Muscle Cells

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Abstract—The mechanisms by which reactive species (RS) participate in the development of atherosclerosis remain incompletely understood. The present study was designed to test the hypothesis that RS produced in the vascular environment cause mitochondrial damage and dysfunction in vitro and, thus, may contribute to the initiating events of atherogenesis. DNA damage was assessed in vascular cells exposed to superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite. In both vascular endothelial and smooth muscle cells, the mitochondrial DNA (mtDNA) was preferentially damaged relative to the transcriptionally inactive nuclear *β-globin* gene. Similarly, a dose-dependent decrease in mtDNA-encoded mRNA transcripts was associated with RS treatment. Mitochondrial protein synthesis was also inhibited in a dose-dependent manner by $ONOO^-$, resulting in decreased cellular ATP levels and mitochondrial redox function. Overall, endothelial cells were more sensitive to RS-mediated damage than were smooth muscle cells. Together, these data link RS-mediated mtDNA damage, altered gene expression, and mitochondrial dysfunction in cell culture and reveal how RS may mediate vascular cell dysfunction in the setting of atherogenesis. (*Circ Res.* **2000;86:960-966.**)

Key Words: reactive oxygen species ■ reactive nitrogen species ■ atherosclerosis ■ oxidative damage

Although reactive species (RS) serve as second messengers in vascular cells to mediate short-term signaling events,[1] the long-term effects of these radicals induce cellular damage and may potentiate atherosclerotic lesion formation. Numerous studies have linked excess RS generation with vascular lesion formation and functional defects.[2,3] Increased levels of oxidatively modified proteins and lipoproteins, including oxidized LDL, lipid peroxidation products, nitrated and chlorinated tyrosines, and advanced glycation end products, have been identified in patients with coronary artery disease.[4–6] A role for RS in atherogenesis is also supported epidemiologically by the association between the common risk factors for myocardial infarction—hypertension, hypercholesterolemia, cigarette smoking, and diabetes mellitus—and increased levels of RS.[7–9]

The RS thought to be important in atherosclerosis include superoxide ($O_2^{\cdot-}$), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), and nitric oxide ($^{\cdot}NO$).[10] Within the arterial wall, metabolic processes, cytokines, and inflammatory responses generate RS in different cell types through multiple and, in some cases, cell type–specific pathways. For example, the oxidative modification of LDL occurs in monocytes only if these cells are activated to express lipoxygenase or induce a respiratory burst with concomitant generation of RS.[11] In this case, the cytoplasmic membrane-associated NADPH oxidase of monocytes/macrophages generates RS derived from $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}NO$. The plasmalemmal NADH/NADPH oxidase and mitochondria are key sources of RS in heart[12] and vascular tissues.[13–15] Whereas RS generation is possible at multiple sites within the mitochondrial respiratory chain,[16] $O_2^{\cdot-}$ generation occurs preferentially at the level of coenzyme Q (UQ), through the accumulation of free radical semiquinone anion species ($UQ^{\cdot-}$) and electron donation from $UQ^{\cdot-}$ to O_2.[17] Consistent with this hypothesis, studies have shown that mitochondrial RS generation continues in the presence of rotenone (a complex I inhibitor) as a result of the supply of electrons via complex II (succinate). Antimycin A, which blocks the formation of the $UQ^{\cdot-}$ at the matrix face and results in the accumulation of $UQ^{\cdot-}$ on the cytoplasmic face, causes increased $O_2^{\cdot-}$ formation[16,18]; H_2O_2 generated via $O_2^{\cdot-}$ dismutation (superoxide dismutase [SOD]) reacts in the presence of transition metals (via the Fenton reaction) to yield reactive perferryl and hydroxyl radicals. Alternatively, $O_2^{\cdot-}$ can react with $^{\cdot}NO$ at near diffusion-limited rates (10^9 $[mol/L]^{-1}$ $seconds^{-1}$)[19] to form $ONOO^-$ that is directly oxidizing or, on protonization, can react similarly to hydroxyl radical.[20]

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In the present study, human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) were treated with RS to test the hypothesis that they induce mitochondrial damage in vascular cells. Mitochondrial injury was reflected by DNA damage, changes in gene expression, protein synthesis, and redox function. It was observed that mitochondrial DNA (mtDNA) was preferentially damaged in both cell types, although HUVECs appeared most sensitive to RS treatment. Decreases in mitochondrial RNA (mtRNA) transcripts, mitochondrial protein synthesis, cellular ATP levels, and mitochondrial redox potential were also observed. These results demonstrate RS-mediated mitochondrial damage in vascular cells and suggest a mechanism by which RS induce vascular cell dysfunction, leading to atherogenesis and other chronic vascular diseases.

Materials and Methods

Cell Culture

HASMCs were purchased from Clonetics and were maintained as described previously.[21] HUVECs were cultured under conditions similar to HASMCs, except in M199 and 20% heat-inactivated FCS. Monolayer cultures in 60-mm plates were treated with various RS at 70% to 80% confluence in serum-free, phenol red–free MEM at 37°C, between passages 5 and 7. After treatment, cells were washed with cold PBS and harvested immediately.

RS Treatments

H_2O_2 and $ONOO^-$ were prepared as described.[22,23] Xanthine oxidase (XO; 5 mU/mL) plus lumazine (LZ; 100 μmol/L) and spermine NONOate (85 μmol/L) were used to generate $O_2^{\cdot-}$ (2 μmol/L min^{-1}) and $^{\cdot}NO$ (0.5 μmol/L min^{-1}), respectively. 3-Morpholinosydnonimine hydrochloride (SIN-1; 1 mmol/L, Molecular Probes) was used to generate both $O_2^{\cdot-}$ and $^{\cdot}NO$ (3.7 to 7 μmol/L min^{-1}).[24]

DNA Isolation and Quantitative Polymerase Chain Reaction (QPCR)

DNA extraction, QPCR conditions, and calculation of DNA lesion frequencies have been previously described.[22] QPCRs were performed in a GeneAmp PCR system 2400 with the GeneAmp XLPCR kit (Perkin-Elmer-Cetus).

Northern Blot Analyses

Total cellular RNA and Northern analyses were performed using standard techniques.[25] mtRNA probes were made by PCR using purified mtDNA template (16S rRNA, sense primer, 2005 to 2022, and antisense primer, 2982 to 3001; NADH dehydrogenase 2 [ND2], sense primer, 4831 to 4847, and antisense primer, 5464 to 5481; and cytochrome b [cyt b], sense primer, 14730 to 14749, and antisense primer, 15845 to 15863) and were gel purified before random [α-^{32}P]dCTP labeling. The levels of RNA in each lane were normalized by hybridization with a *β-actin* probe.

Mitochondrial Protein Synthesis

Mitochondrial protein synthesis analyses were conducted as previously described.[26] Equal amounts of total protein (50 μg) were run on 10% to 20% gradient SDS-PAGE gels. The percentage labeling of translation products was determined by densitometry of all bands for treated and untreated samples. The sum of the labeled bands for each sample was used to calculate relative levels of incorporation.

MTT and ATP Assay

Cells were seeded in 96-well plates at a density of 8000 cells per well and incubated at 37°C for 48 hours. Cells were then treated with RS for 1 hour, washed with PBS, and incubated in conditioned medium for 1 hour with 2.0 μg/mL MTT and lysed, and absorbance was measured at 570 nm.[27] Total cellular ATP content was determined by using an ATP determination kit (Molecular Probes, A-6608) and a MicroLumat Plus LB (EG&G Berthold) microinjector luminometer.

Rhodamine (R123) Fluorescence and $O_2^{\cdot-}$ Production

After 1 hour of RS treatment, cells were trypsinized, washed, pelleted, and resuspended in culture medium containing 1 μmol/L R123 (37°C). R123 fluorescence was determined using a Becton Dickinson FACS flow cytometer. To determine $O_2^{\cdot-}$ production, aconitase activity was performed,[28] with minor modifications for cultured cells. Cells were treated with H_2O_2 for 1 hour and washed with PBS, and aconitase activity was measured for 1 to 60 minutes. NADPH production was normalized to total protein.

Results

RS Induce Vascular mtDNA Damage

Vascular cell DNA damage was assessed using a well-validated, sensitive QPCR assay that relies on the principle that oxidative DNA lesions inhibit DNA polymerases (eg, *Taq* and *rTth* DNA polymerase). DNA damage was quantified by comparing the relative efficiency of amplification of large fragments of DNA (16.2 kb for the mtDNA; 17.7 kb for the *β-globin* gene) and normalizing this to the amplification of smaller (<250-bp) fragments, which have a statistically negligible likelihood of containing damaged bases.

Treatment with H_2O_2 (0.2 mmol/L) for 1 hour resulted (see Table and Figure 1A) in damage to both genomes in HUVECs ($P<0.005$). Similarly, mtDNA damage occurred in H_2O_2-treated HASMCs ($P<0.005$); however, no significant nDNA damage was observed. Time course analysis in HUVECs and HASMCs demonstrated that mtDNA damage accumulated rapidly, within 10 and 15 minutes, respectively. The nDNA *β-globin* locus accumulated DNA damage less rapidly (Figure 1B), requiring 1 hour of exposure before nDNA damage was observed in HUVECs.

$ONOO^-$ treatment (0.1 mmol/L and 0.5 mmol/L) caused significant mtDNA damage in HUVECs ($P<0.005$), whereas only 0.5 mmol/L $ONOO^-$ resulted in HASMCs mtDNA damage ($P<0.005$, Table). Lower doses of $ONOO^-$ (0.05 mmol/L; data not shown) caused mtDNA damage 30 minutes after treatment in HUVECs (0.17 lesions per 10 kb, $P=0.041$), which dissipated by 60 minutes (0.01 lesions per 10 kb, $P=0.71$), implying that the damage had been repaired. HASMCs did not sustain significant damage with low-dose $ONOO^-$ treatment (0.13 lesions per 10 kb at 30 minutes, $P=0.069$; 0.00 lesions per 10 kb at 60 minutes, $P=0.97$). No significant levels of nDNA damage were observed in either cell type (Table).

RS-Generating Systems Induce DNA Damage

To examine the effects of sustained, low-dose generation of RS, HUVECs and HASMCs were treated with 5 mU/mL of XO plus LZ (generates ≈2 μmol/L min^{-1} $O_2^{\cdot-}$) and/or 85 μmol/L spermine NONOate (generates ≈0.5 μmol/L min^{-1} $^{\cdot}NO$) to reveal the contributions of $O_2^{\cdot-}$, H_2O_2, $^{\cdot}NO$, and $ONOO^-$ to damage. Finally, cells were treated with platelet-derived growth factor (PDGF), which causes endogenous RS production.[1]

HUVECs exposed to 5 mU/mL XO plus LZ had increased mtDNA damage ($P<0.05$, Table), which was inhibited by SOD or catalase (not shown), implicating both H_2O_2 and $O_2^{\cdot-}$ (XO plus LZ generates both radicals) in mtDNA damage. To assess the influence of $^{\cdot}NO$, cells were treated with a combination of

Lesions After Oxidant Exposure

	Estimated Lesion Frequency				
	Untreated Control	0.2 mmol/L H_2O_2	0.1 mmol/L $ONOO^-$	0.5 mmol/L $ONOO^-$	Decayed $ONOO^-$
HASMCs					
mtDNA	0	1.68 (0.25)*	0.16 (0.05)	1.42 (0.35)*	0
nDNA	0	−0.09 (0.14)	−0.08 (0.05)	−0.04 (0.03)	ND
HUVECs					
mtDNA	0	2.59 (0.33)*	0.19 (0.03)*	2.81 (0.37)*	0.07 (0.03)
nDNA	0	1.02 (0.11)*	0.07 (0.10)	1.05 (0.40)	0.01 (0.05)
	Estimated Lesion Frequency				
	Untreated	$O_2^{\cdot-}+H_2O_2$	$O_2^{\cdot-}$,H_2O_2+NO	SIN-1	SIN-1+SOD
HASMCs					
mtDNA	0	0	0	0.13 (0.05)	0.05 (0.15)
nDNA	0	0	0	ND	ND
HUVECs					
mtDNA	0	0.22 (0.04)†	0.41 (0.04)*	1.71 (0.33)*	−0.17 (0.12)
nDNA	0	0	0.08 (0.11)	ND	ND
	mtDNA Lesion Frequency				
	Untreated Control	5 ng/mL PDGF			
HASMCs					
30 minutes	0	0.41 (0.41)			
60 minutes	0	0 (0.42)			
HUVECs					
30 minutes	0	0.76 (0.01)*			
60 minutes	0	0.55 (0.54)			

Top, Estimated lesion frequency (per 10 kb) was calculated for mtDNA and nDNA of RS-treated cells compared with untreated cells. Decayed $ONOO^-$ represents the NO_2^- and NO_3^- content of 0.5 mmol/L $ONOO^-$ after incubation for 1 hour. Middle, Estimated frequency of lesions (per 10 kb) in mtDNA and nDNA after treatment with XO+LZ ($O_2^{\cdot-}$ and H_2O_2 generation), spermine NONOate (NO generation), SIN-1 (a source of NO and $O_2^{\cdot-}$), SOD+SIN-1 (cells were pretreated with 300 U/mL SOD). Bottom, mtDNA lesion frequencies (per 10 kb) were calculated from cells treated with 5 ng/mL PDGF for 30 and 60 minutes compared with untreated cells. Values are mean±SEM.

ND indicates no data; a negative value (−) indicates less damage compared with untreated controls.

*Significantly different from control ($P<0.005$); †significantly different from control ($P<0.05$).

85 μmol/L spermine NONOate and 5 mU/mL XO plus LZ. NO exposure alone did not cause DNA damage (not shown), but the addition of XO-derived $O_2^{\cdot-}$ and H_2O_2 caused mtDNA damage in HUVECs ($P<0.05$, Table). Similarly, SIN-1 treatment (1 mmol/L; generates ≈3.7 to 7 μmol/L min^{-1} $O_2^{\cdot-}$ and NO) caused significant mtDNA damage in HUVECs ($P<0.001$, Table and Figure 2). As with other treatments, HASMCs were less affected by RS than HUVECs were (Table, Figure 2).

To assess the effects of endogenous RS, we determined the levels of mtDNA damage in HUVECs and HASMCs at 30 and 60 minutes after 5 ng/mL PDGF treatment. Similar to the 0.05 mmol/L $ONOO^-$ treatment, HUVEC mtDNA damage was increased 30 minutes after PDGF treatment (Table; $P<0.001$). However, no damage was observed at 60 minutes, indicating that the damage was transient (Table). No significant differences were observed in PDGF-treated HASMCs. Together, these data reveal that HUVECs mtDNA appear more susceptible to both exogenous and endogenous RS-mediated damage than do HASMCs.

Because RS increased mtDNA damage in both HUVECs and HASMCs, their influence on mtRNA transcript levels, mitochondrial protein production, and mitochondrial redox potential was investigated. $ONOO^-$ was chosen on the basis of the data reported here and its documented production in the vasculature and atherosclerotic lesions.[29] Treatment with 0.5 mmol/L $ONOO^-$ resulted in up to a 55% decrease in ND2 and *cyt* b transcripts in HASMCs, and a 45% to 50% reduction in ND2 and *cyt* b transcripts in HUVECs (Figure 3). Treatment with 0.1 mmol/L $ONOO^-$ reduced ND2 and *cyt* b transcript levels by 25% to 30% in HUVECs and 5% to 15% in HASMCs (Figure 3).

RS Alter Mitochondrial Protein Synthesis

Having demonstrated that $ONOO^-$ exposure decreased mtRNA levels, the influence of $ONOO^-$ on mitochondrial protein synthesis was assessed by measuring [^{35}S]methionine incorporation in the presence of emetine, an inhibitor of nuclear, but not mitochondrial, protein synthesis. Exposure of cells to $ONOO^-$ (0.5 mmol/L) resulted in a substantial decrease in mitochondrial protein synthesis (Figure 4). Lower doses of $ONOO^-$ (0.1 mmol/L) produced a 12% decrease in [^{35}S]methionine incorporation in HASMCs (not performed in

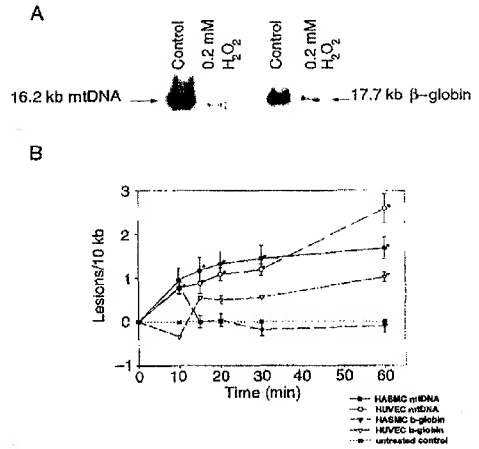


Figure 1. RS-induced mtDNA and nDNA damage. A, HUVECs were exposed to 0.2 mmol/L H_2O_2 for 1 hour and QPCR was performed. Control cultures were incubated in serum-free medium alone. B, Time course of H_2O_2-induced HASMC and HUVEC injury. Cells were treated with 0.2 mmol/L H_2O_2 for 0 to 60 minutes, and lesions per 10 kb were estimated. Data are mean levels±SEM. *Significantly different ($P<0.05$) damage relative to untreated cells.

HUVECs). Treatment with H_2O_2 (0.2 mmol/L) resulted in a 23% and 33% decrease in overall mitochondrial protein synthesis in HUVECs and HASMCs, respectively.

RS Inhibit Mitochondrial Function

The influence of RS on mitochondrial redox potential and cellular ATP level was evaluated by measuring MTT reduction and cellular ATP content. Analysis of MTT reduction by succinate dehydrogenase (mitochondrial complex II) reflects net cellular respiratory and redox functions (Figure 5).[30] Exposure to 0.1 mmol/L $ONOO^-$ did not significantly decrease MTT reduction or cellular ATP, whereas 0.5 mmol/L $ONOO^-$ induced a significant decrease in MTT reduction and cellular ATP in each

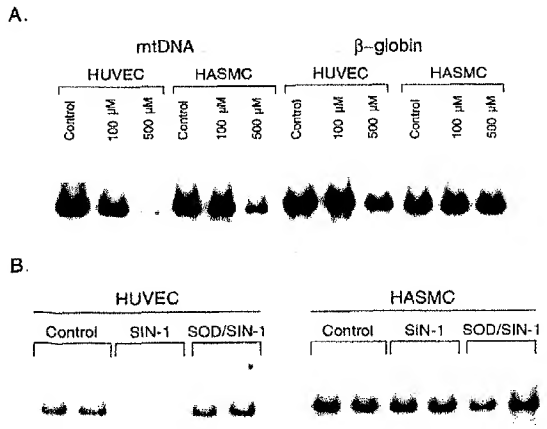


Figure 2. Peroxynitrite-induced mtDNA and nDNA damage in HUVECs and HASMCs. A, Cells were treated for 1 hour with 0.1 or 0.5 mmol/L $ONOO^-$, and QPCR was performed. Control cultures were incubated in serum-free medium alone. B, HUVECs or HASMCs were treated with 1 mmol/L SIN-1, in absence or presence of 300 U/mL SOD.

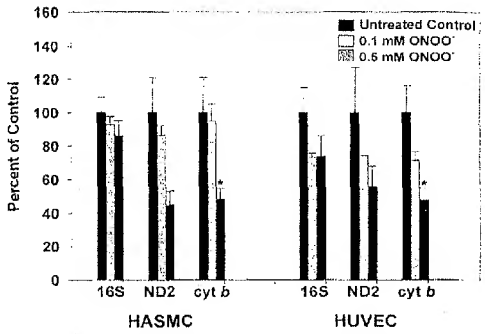
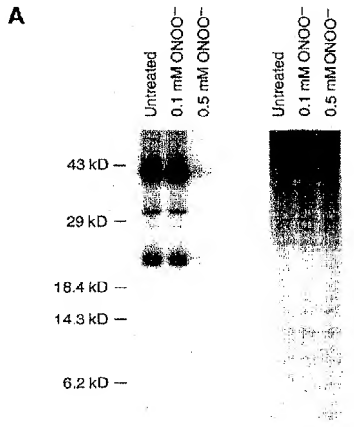


Figure 3. mtRNA transcript levels in $ONOO^-$-treated cells. Data are mean relative transcript levels (±SEM) normalized to *β-actin* for 16S rRNA, ND2, and *cyt* b after exposure to 0.1 or 0.5 mmol/L $ONOO^-$ for 1 hour, compared with untreated cells. Transcript levels were determined by Northern analysis and hybridization to the appropriate radiolabeled probe. *Transcript levels significantly different ($P<0.05$) from controls.

cell type ($P\leq0.05$). Treated HUVECs had greater declines in cellular ATP and MTT reduction compared with HASMCs (ATP, $P=0.002$; MTT, $P=0.006$). To verify that the decreases in MTT reduction and ATP production were not due to cell death, cells were stained with trypan blue 1 hour after oxidant exposure, showing essentially no cell death (<5% trypan blue staining) at times of MTT reduction and ATP level assessment.

To evaluate whether mitochondrial membrane potentials ($\Delta\psi$) were altered by RS treatments, R123 uptake was determined in cells 1 hour after RS treatment. R123 selec-



B

^{35}S-Methionine Incorporation (% of control)

	Control	0.2 mM H_2O_2	0.1 mM $ONOO^-$	0.5 mM $ONOO^-$
HASMC	100	67	88	30
HUVEC	100	77	ND	45

Figure 4. [^{35}S]Methionine incorporation in mitochondrial synthesized proteins. A, Protein labeling after exposure to 0.1 or 0.5 mmol/L $ONOO^-$ in HASMCs (left). Right, Same gel stained with Coomassie blue (total protein). B, Relative percentages, compared with controls, of [^{35}S]methionine incorporation in both HUVECs and HASMCs exposed to 0.2 mmol/L H_2O_2 and 0.1 or 0.5 mmol/L $ONOO^-$. ND indicates no data.

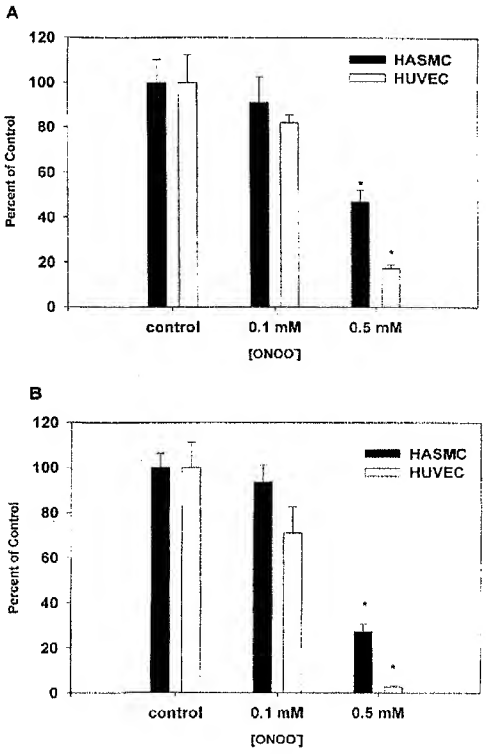


Figure 5. Inhibition of mitochondrial complex II reduction and cellular ATP content. HASMCs and HUVECs were exposed to 0.1 or 0.5 mmol/L $ONOO^-$ for 1 hour and analyzed for relative levels of MTT reduction (A) and cellular ATP content (B). *Levels significantly different ($P<0.05$) from controls. Values are expressed relative to untreated cells (100%).

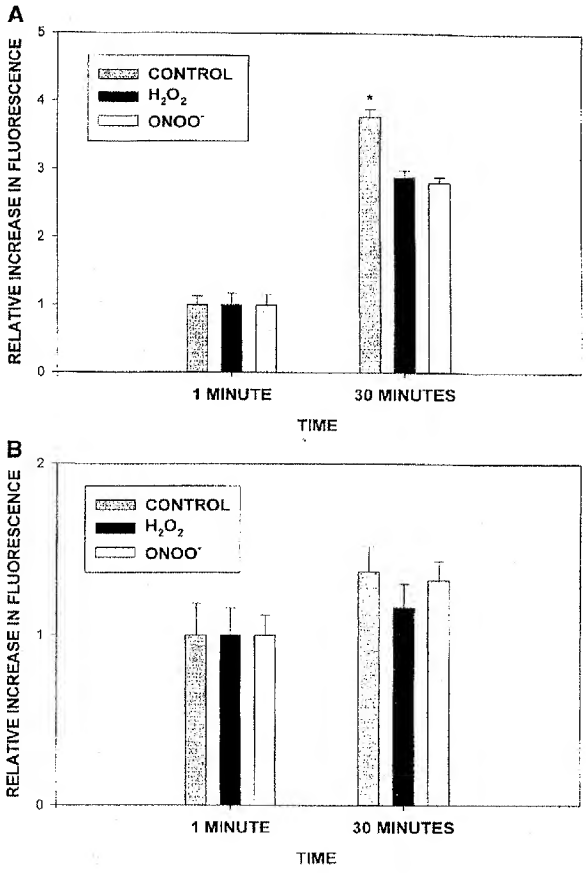


Figure 6. R123 uptake in HUVECs (A) and HASMCs (B). Cells were treated with 0.2 mmol/L H_2O_2 or 0.5 mmol/L $ONOO^-$ in serum-free medium for 1 hour, trypsinized, washed, and resuspended in conditioned medium containing 1 μmol/L R123. Fluorescence was assessed at 1 minute and 30 minutes after R123 addition. Increased fluorescence is expressed relative to the 1-minute time point. *Significantly different ($P<0.05$) level of fluorescence.

tively accumulates in the mitochondria, and rate of uptake is an indication of $\Delta\psi$. HUVECs treated with 0.2 mmol/L H_2O_2 or 0.5 mmol/L $ONOO^-$ had significantly lower rates of R123 uptake (Figure 6). In contrast, no differences were observed in R123 uptake in HASMCs (Figure 6).

To assess secondary RS formation associated with the treatments, we determined aconitase activity in HUVECs 1 to 2 hours after 0.2 mmol/L H_2O_2 treatment. Aconitase is specifically inactivated by $O_2^{\cdot-}$ and $ONOO^-$[28] and is unaffected by H_2O_2 (Figure 7), so that aconitase activity is inversely related to the generation of $O_2^{\cdot-}$ and $ONOO^-$. HUVECs treated with 0.2 mmol/L H_2O_2 had a 3.4-fold decrease in aconitase activity compared with controls (Figure 7), indicating an increased production of secondary $O_2^{\cdot-}$ and $ONOO^-$ in the treated cells.

Discussion

The RS used in this study mediate mitochondrial damage and dysfunction in HUVECs and HASMCs in vitro by increasing mtDNA damage, decreasing mtRNA transcripts, inhibiting mitochondrial protein synthesis, reducing cellular ATP, and altering redox function. These data suggest that RS, in pathophysiologically relevant concentrations, mediate mitochondrial damage and dysfunction that affect vascular cell function and can contribute to chronic vascular diseases.

With the exception of exposure to NO, the various RS used, at their selected rates of production and/or concentration, yielded similar extents of mtDNA damage. This is likely due to the often similar target molecule reactions of different RS (eg, $O_2^{\cdot-}$ + H_2O_2 versus $ONOO^-$) as well as to their broad and potent reactivities. This is of relevance in atherogenesis, as individual RS will be generated at different rates by vascular cells during disease progression. The concentrations of $ONOO^-$ used within these studies were well within physiological ranges.[20] Administration of a bolus concentration of $ONOO^-$ produces effects comparable with longer, sustained low-dose exposures[31]; the additions of $ONOO^-$ in the present study were equivalent to exposure to 1 μmol/L $ONOO^-$ for 3 to 13 minutes. The half-life of $ONOO^-$ at pH 7.4 is 1.6 seconds; thus, remotely located secondary oxidative reactions likely contribute to the sustained effect of this relatively long-lived oxidant.

mtRNA transcripts declined subsequent to $ONOO^-$ exposure in HASMCs and HUVECs. This reduction was transient; 2 hours after treatment, mtRNA levels were restored to the same

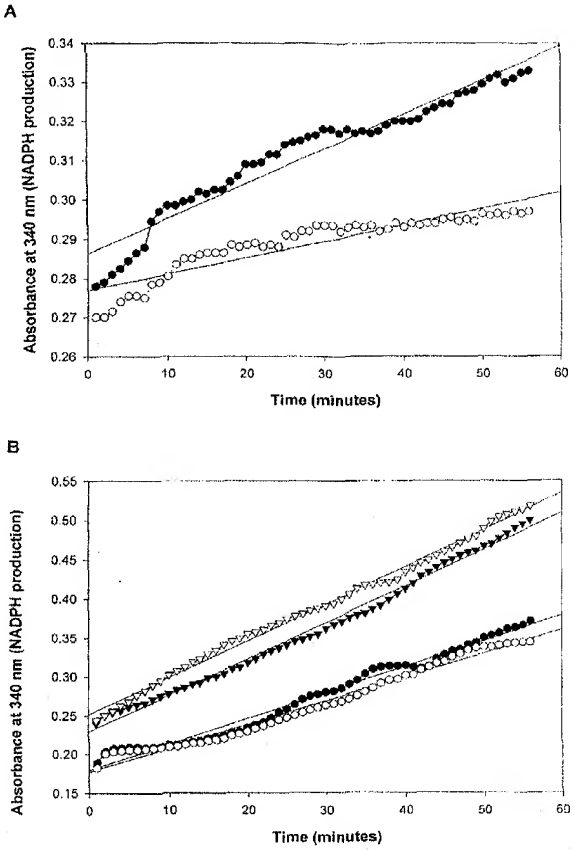


Figure 7. Aconitase activity in H_2O_2-treated HUVECs. A, Control (no RS, 1 hour; ●) and treated (0.2 mmol/L H_2O_2, 1 hour; ○) cells were washed in PBS and lysed in assay buffer. Aconitase activity was measured for 1 hour. Decreased activity indicates increased $O_2^{\cdot-}$ production. B, Purified aconitase (5 mU, △, ▲; 2 mU, ○, ●) treated with serum-free medium (●, ▲) or serum-free medium plus 0.2 mmol/L H_2O_2 (○, △).

level as controls, indicating that mtRNA transcript reduction was directly related to RS exposure and reversible cell injury. The observation that the mitochondrial 16S rRNA transcript is minimally affected by exposure to RS may be the result of its previously noted differential expression[32] or transcript stability.[33]

Consistent with alterations in mitochondrial function, protein synthesis, and redox potential (complex II), mitochondrial membrane potentials were reduced in RS-treated HUVECs. In contrast, R123 uptake was not significantly affected in HASMCs, potentially because of increased resistance to RS-mediated damage (due to potentially greater antioxidant capacity or repair processes), and/or because of unique cell characteristics. Studies in liver tissues have shown that whereas $ONOO^-$ treatment inhibits electron transport, it also promotes Ca^{2+} release from rat liver mitochondria under certain conditions, with maintenance of $\Delta\psi$[34]; it may be that HASMCs respond similarly to RS-mediated stress. Further studies of the antioxidant and repair capacities of HASMCs relative to HUVECs will be required to determine why HASMCs appear more resistant to RS-mediated damage. Nevertheless, taken together, these data indicate that oxidative injury can mediate a cascade of events in HUVECs and HASMCs that ultimately result in mitochondrial damage and function.

A consistent observation for HUVECs and HASMCs was that nDNA is less affected by RS than mtDNA. It is possible that the concentrations of exogenously added RS achieved in the nucleus were lower than in the mitochondria. However, it is more likely that differences in structure, protein content, and repair make the nDNA more resistant to oxidative damage. The mtDNA is sensitive to damage because it lacks both protective histone and nonhistone proteins and has a limited DNA repair capacity (relative to the nucleus). For example, the mtDNA accumulates significantly higher levels of the DNA oxidation product 8-hydroxydeoxyguanosine than does the nDNA.[35] In addition, the high lipid-to-DNA ratio in mitochondria makes them especially susceptible to lipophilic species, whereas the attachment of the mtDNA to the matrix side of the inner membrane also increases sensitivity to membrane disturbances and makes mitochondria a target for electrophiles generated within the membrane.[36] Although endogenous rates of mitochondrial and nuclear radical generation are similar,[37] the added capacity of mitochondria to generate $^{\cdot}NO$[38] or differences in antioxidant defense system activities may contribute to the sensitivity of these organelles. Because the amount of intact DNA present at any point in time reflects a balance between damage and repair, it is possible that the extensive and persistent mtDNA damage is due to lesser repair and/or increased generation of secondary RS due to mitochondrial injury.

Although our studies have focused on the effects of RS on mitochondrial function, mitochondria themselves serve as a major source of RS. Mitochondria consume more than 90% of the oxygen available to the cell; 1% to 3% is used in the production of $O_2^{\cdot-}$.[39] In addition to $O_2^{\cdot-}$, mitochondria produce $^{\cdot}NO$[40] and CO_2[41] and, thus, are capable of generating both intramitochondrial reactive oxygen species and reactive nitrogen species (ie, $ONOO^-$ and $ONOOCO_2^-$), which efficiently mediate secondary oxidation and nitration reactions.[42] Several reports reveal that $ONOO^-$ inhibits mitochondrial respiration and enhances rates of respiratory chain $O_2^{\cdot-}$ and H_2O_2,[43] implicating these species in chronic mitochondrial damage. Consistent with this notion, numerous studies have shown an age-dependent decrease in mitochondrial respiratory enzyme activities,[40] increased lipid peroxidation,[44] mtDNA mutation,[45,46] and damage. Studies have also shown that oxidized LDL inhibits mitochondrial function[47] and induces production of mitochondrial antioxidants[48] and that mitochondrial RS generation is involved in LDL oxidation.[14] Finally, RS are capable of inhibiting specific mitochondrial enzymes,[49,50] affecting cellular antioxidant and energetic capacities. Consequently, chronic and age-related increases in RS production will cause mitochondrial damage and dysfunction that perpetuate a catastrophic cycle of cellular injury, further RS generation, mitochondrial decline, and cell death. We are currently testing this hypothesis utilizing in vivo models of atherosclerosis.

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